Towards the Biosynthetic Pathway of Lesquerolic Acid in *Physaria fendleri*, an Emerging Industrial Crop

Undergraduate Research Thesis

Presented in partial fulfillment of the requirements for graduation *with honors research distinction* in Molecular Genetics in the undergraduate colleges of The Ohio State University

by

Erin Ponting

The Ohio State University
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Project Advisor: Dr. Ana P. Alonso, Department of Molecular Genetics

Abstract

Physaria fendleri (Lesquerella) is a Brassicaceae that produces the hydroxy fatty acid, lesquerolic acid. This invaluable compound has many industrial applications such as cosmetics, greases, plastics, and biofuels. In the United States, the main source of hydroxy fatty acids is ricinoleic acid, from the castor plant (*Ricinus communis*). Due to ricin, a highly toxic compound, the United States has banned commercial growth of this plant and the entirety of our supply must be imported. On the other hand, Physaria does not produce toxic compounds and lesquerolic acid is only two carbons longer than ricinoleic acid, resulting in the same chemical properties.

Physaria fendleri, as a noncompetitive winter annual, is a well suited substitute crop for castor. However, fatty acid production needs to be increased for it to be economically viable. In order to enhance hydroxy fatty acid production, the biosynthetic steps need to be identified, specifically the one at which the hydroxyl group is added. To tackle this question, chemical inhibitors that are specific to the enzymes involved in fatty acid synthesis were utilized. Thiocarbamates have been shown to be effective inhibitors of fatty acid elongation, which takes place in the endoplasmic reticulum (ER). In this study, S-ethyl dipropylcarbamothioate (EPTC) and Diallate, both thiocarbamates, were tested to unravel whether the hydroxyl group was added to the fatty acid before or after elongation.

I-Background

Physaria fendleri (syn. Lesquerella) is a Brassicaceae whose seeds produce lesquerolic acid, a valuable hydroxy fatty acid (HFA) that could be used in various industrial applications such as cosmetics, lubrications, paints, plastics, and biofuels (Kim et al., 2011, Cocuron et al., 2014; **Figure 1**)

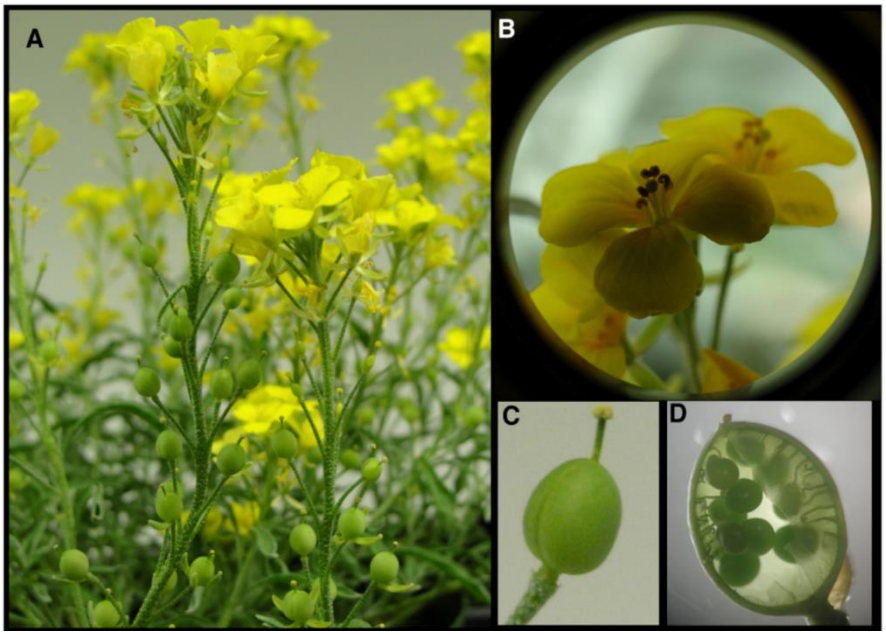


Figure 1. ***Physaria fendleri* plant anatomy.** (A) Mature flowering plant; (B) Flowers; (C) Pod; (D) Half pod with seeds. From Cocuron *et al*., (2014)

Currently, ricinoleic acid, extracted from the castor plant (*Ricinus communis*), is the main source of HFAs. However, the United States has banned commercial growth of this tropical crop for the extraction of ricinoleic acid because it also produces highly toxic compounds such as ricin, and causes allergic reactions (Ogunniyi, 2006). Therefore, the entirety of our HFA supply has to be imported from producing countries such as India, Brazil, and China (obtained from Linnaeus Plant Sciences Incorporated, http://www.linnaeus.net). Physaria, on the other hand, is native to the United States, free of toxins, and would therefore be a promising alternative to castor oil. Indeed, lesquerolic acid has been shown to have similar chemical structure and properties to ricinoleic acid (Cermak et al., 2006; **Figure 2**). Although there is a difference of two carbons

between the chemical structures of lesquerolic acid and ricinoleic acid, the hydroxy site allows for esterification that can yield the same biodiesel additive estolides. These estolides from Physaria and castor are biodiesel additives that show the best low temperature properties in comparison to products sold commercially (Cermak et al., 2006). Besides the valuable HFAs, Physaria seeds also produce a natural gum that can be used as a food or industrial thickener (Abbott et al., 1994, Wu and Abbott, 1996). There is also a favorable distribution of essential amino acids comparable to soybean meal that makes the seeds an ideal protein supplement for livestock (Carlson et al., 1990, Wu and Hojilla-Evangelisa, 2005). Finally, with its versatility and ability to be grown as a winter annual in the southwestern United States, Physaria is a promising dedicated industrial crop that can be placed in rotation with our current commodity crops (Brahim et al., 1996, Dierig et al., 2011).

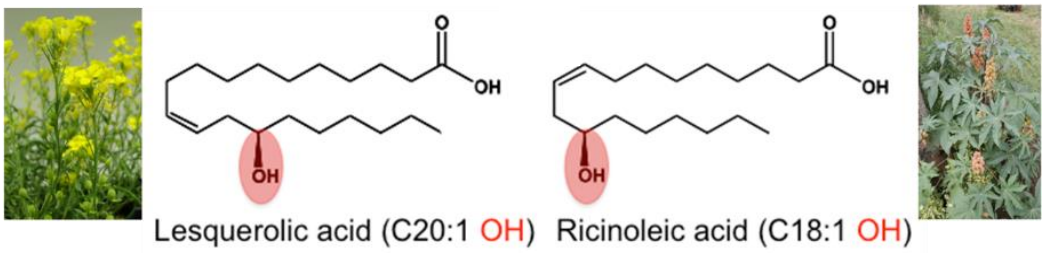


Figure 2. Lesquerolic acid (C20:1 OH) from *Physaria fendleri* in comparison to ricinoleic acid (C18:1 OH) from *Ricinus communis.*

Naturally, Physaria seeds contain approximately 25% oil (w/w), of which 60% is lesquerolic acid (Barclay, 1962, Isbell et al., 2008). In order to make this crop economically viable, total oil and HFA contents must be improved. The long-term goal of this project is to optimize the production of HFAs in Physaria. To date, there is a lack of knowledge about the mechanisms leading to HFA synthesis and accumulation. A metabolomics study in developing embryos was conducted in the Alonso Lab, and revealed the main metabolic pathways that were

active during oil synthesis (Cocuron et al., 2014). Glucose and glutamine were found to be the main sources of carbon and nitrogen that the developing embryos receive from the plant. Malate and citrate were the main organic acids in the embryos, which lead to the possibility that they are the main precursors for fatty acid synthesis and elongation. A subsequent transcriptomic analysis demonstrated that at least 20 genes involved in lipid synthesis appeared to have co-evolved in Physaria embryos (Horn et al., 2016). This co-evolution is thought to channel HFAs into storage lipids (triacylglycerols). To refine and quantify the biochemical pathways involved in HFA production, the Alonso Lab has developed and validated culture conditions that mimic the development of the embryos *in planta* (unpublished results). This will allow: i) feeding the embryos with ^{13}C-labeled substrates to follow the flow of labeled carbon through the primary metabolic pathways; and ii) testing inhibitors specific to enzymes involved in fatty acid synthesis to identify the step at which the hydroxyl group is added.

In plants, fatty acid synthesis and elongation takes place in the plastid and the endoplasmic reticulum, respectively. Within the plastid, pyruvate is utilized to generated acetyl-CoA, the essential two-carbon compound that is the main building block of fatty acid synthesis. Through activation of the enzyme acetyl CoA-carboxylase, malonyl-CoA is formed and then elongated into fatty acids by incremental two carbon acetyl additions by the enzyme fatty acid synthase (Trenkamp et al., 2004, Fehling et al., 1991). Once fatty acids reach 16 carbons or 18 carbons in length, they can be exported to the endoplasmic reticulum where elongases (β-ketoacyl-ACP synthases) can create very long chain fatty acids such as lesquerolic acid (**Figure 3**).

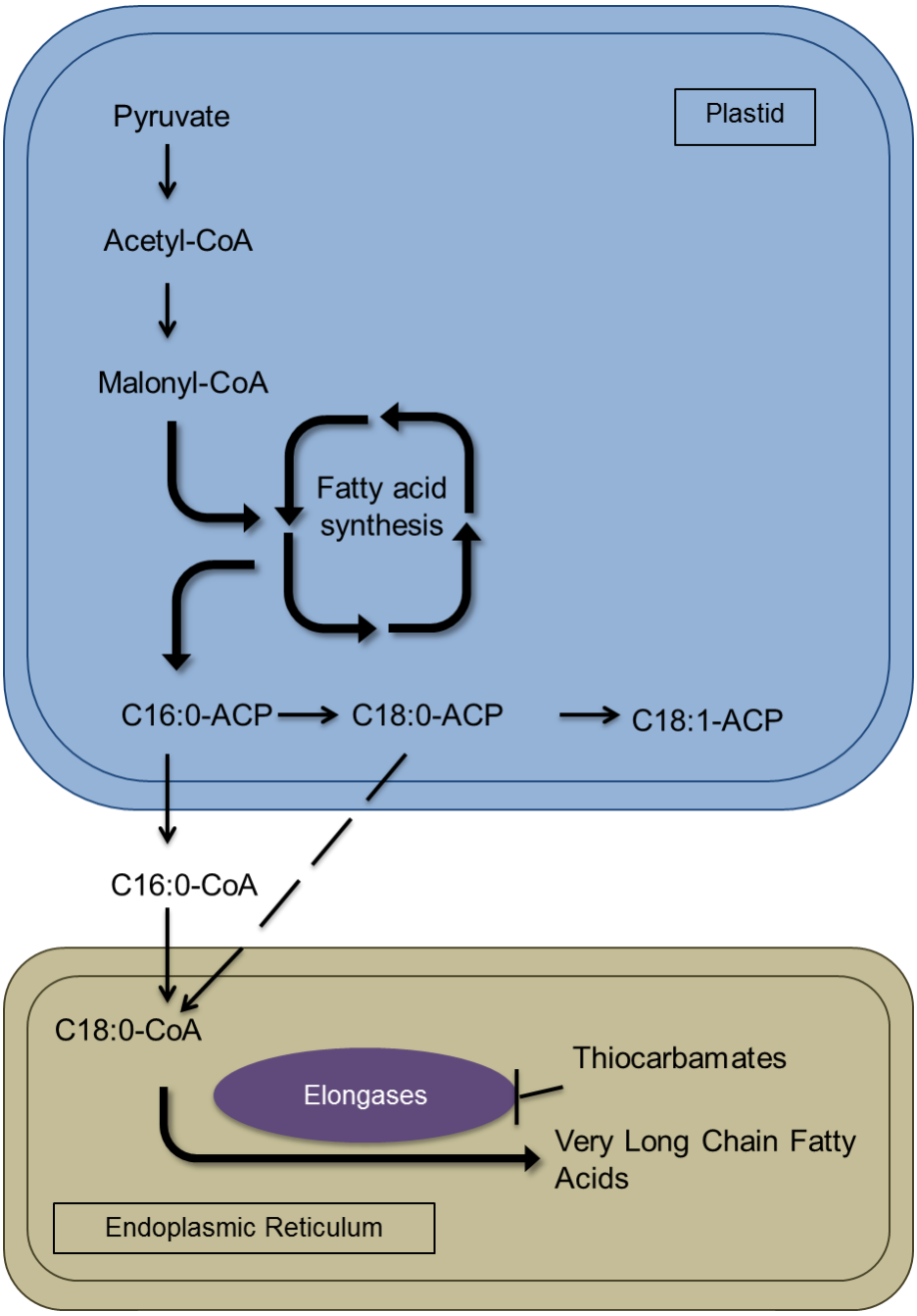


Figure 3. Fatty acid synthesis in plant seeds.

Although the general outline of lesquerolic acid formation is known, the crucial hydroxylation step has not been determined. The hypothesis is that the hydroxyl group in lesquerolic acid is added after the elongation step in developing Physaria embryos. In order to test this hypothesis, thiocarbamates were used as inhibitors of the elongases that are responsible

for adding two carbons to fatty acids that are longer than 18 carbons (Gronwald, 1991). In this study, elongase inhibitors such as S-ethyl dipropylcabamothicate (EPTC) and Diallate were supplemented to embryos in culture to test if the hydroxyl group in lesquerolic acid is added before or after the elongation step in oil synthesis. If hydroxylation occurs before elongation, lesquerolic acid should decrease whereas other forms of hydroxylated fatty acids with shorter chains will be detected.

II-Materials and Methods

2.1- Chemicals:

The 3 N methanolic/hydrochloric acid and toluene used for fatty acid extraction and methylation as well as C17:0 standard (triheptadecanoin) were obtained from Sigma Aldrich (St. Louis, MO). Solvents for gas chromatography-mass spectrometry (GC-MS) and chemical inhibitors (EPTC and Diallate) were purchased from Fisher Scientific (Pittsburgh, PA).

2.2- Plant Growth:

Physaria seeds were planted in 18-cm pots with an autoclaved soil/vermiculite mixture accompanied by time release fertilizer and pesticide. The top of each pot was plastic wrapped to promote constant moisture conditions. Plants were grown at 22°C with a light intensity of 320 μmol per m^2 per second during a 16-h/8-h day/night photoperiod. When sprouting was observed, the plastic wrap was removed. Once initial bud development was prevalent on one plant, all other plants were removed from the pot to prevent competition for root growth and more fertilizer was added. Flowers were hand pollinated daily and new flowers were marked on their first day of pollination by painting the petiole with a color corresponding to the day of the week.

This was done to track embryo development and was designated as Days After Pollination (DAP).

2.3- Embryo Culturing Conditions:

To perform *in vivo* embryo culturing, siliques from Physaria plants were harvested at 18 DAP. A culture medium was prepared with glucose, sucrose, HEPES buffer, glutamine, Murashige & Skoog salts, and Gamborg vitamins. The pH was then corrected to 6.3 and the medium was filtered under sterile conditions using a syringe with a 0.22 micron filter attachment. The rest of the procedure took place under sterile conditions at a clean bench. Siliques were sterilized with 20% bleach. Culture plates with six wells were fitted with two double glass filters per well to prevent anoxia during incubation. Master mixes were prepared that contained the aforementioned culture medium as well as polyethyleneglycol, abscisic acid, and either 1% (200 proof) ethanol or chemical inhibitor. These master mixes were thoroughly vortexed and applied in 1 mL quantities to the designated wells. Embryos were then dissected from the siliques and methodically placed into wells until each well consisted of 8 embryos. The lid was sealed onto the plate with Millipore tape and the plate was allowed to incubate for 9 days at 21^{o}C under constant light 12 µmol per m^2 per second to establish a steady state metabolism. After 9 days, the embryos were collected using a steel-strainer. Upon collecting, the embryos were washed with ddH_2O, surface dried with Kimwipes, and freeze dried with liquid nitrogen in a pre-weighed 2mL- microcentrifuge tube. The samples were then put in a lyophilizer for 3 days for further drying, the dry weight was recorded, and the embryos were stored in a -80^{o}C freezer until extractions could be performed.

2.4- Biomass Characterization of Cultured Embryos*:*

Fatty Acid Extraction: One tungsten bead (5 mm diameter) was added to each tube of dry embryos as well as 50 µL of C17:0 internal standard (triheptadecanoin, 1 mg/mL) and 1 mL of hexane:isopropanol (2:1, v/v). The embryos were disrupted utilizing a bead beater at 30 Hz for 5 minutes. The tubes were centrifuged at 17,000 g for 15 minutes at room temperature. The supernatant was carefully transferred to a separate glass tube (13 x 100 mm) without disturbing the pellet. These steps were repeated two more times, with first 1 mL of hexane:isopronanol (2:1, v/v) and after 0.5 mL. In order to ensure that all the protein and carbohydrates were collected for further analysis, the collected supernatant was centrifuged at 800 g for 5 minutes at 22°C and the resulting pellet was transferred back to the original 2 mL tube using 500 µL of hexane. The combined pellet was dried down under nitrogen and stored at -80°C for later protein quantification. The collected supernatant was dried down under nitrogen for future methylation.

Fatty Acid Methylation: Extracted fatty acids were subjected to methylation: the acyl chains from the triacylglycerols were cleaved from the glycerol backbone and then esterified with methyl groups in order to produce volatile fatty acid methyl esters (FAMEs) that can be analyzed by GC-MS. In order to accomplish this, 150 µL of toluene and 0.5 mL of 3 N methanol/hydrochloric acid was added to each tube and then vortexed. Nitrogen gas was utilized to flush the oxygen from the tubes before sealing them. The samples were incubated at 80°C for two hours, with intermittent vortexing to ensure homogeneity. The reaction was quenched with 250 µL 5% (w/v) sodium bisulfate. In order to separate the extracted fatty acids from the polar layer, 1 mL of hexane was added and the tubes were vortexed and centrifuged at 800 g for 10 minutes at room temperature. 200 µL of the upper phase containing FAMEs was transferred to a vial containing 800 µL of hexane in order to make a suitable dilution for GC-MS analysis.

Protein Extraction: The pellet remaining from fatty acid extraction was subjected to protein extraction. 0.5 mL of protein extraction buffer (20 mM Tris-hydrochloric acid, 150 mM sodium chloride, 1 % sodium dodecyl sulfate, pH of 7.5) was added and the tubes were allowed to vortex for 15 minutes in a 42°C oven. They were then centrifuged at 17,000 g for 10 minutes at room temperature and the supernatant containing the proteins was collected into a separate 1.5 mL microcentrifuge tube. The process was repeated one more time, and the supernatant was combined to ensure all proteins were collected.

Fatty Acid Quantification: Fatty acid content and composition were determined through GC-MS. As described in Tsogtbaatar *et al*, (2015) FAMEs were analyzed using a Thermo Trace gas chromatograph coupled to an ISQ single quadrupole mass spectrometer. FAME derivatives were separated using an Omegawax 250 capillary (30 m × 0.25 mm × 0.25 μm) column at a constant flow rate of 1.4 mL per min. Helium was used as the carrier gas. The GC conditions were as follows: initial temperature was set to 170°C and held for 30 seconds. The oven temperature was then raised to 245°C at 100°C per min and held for 13.75 minutes. The injection temperature was fixed at 225°C and the injection mode set to split with a split ratio of 10. For the MS analysis, the mass spectra were acquired using electron impact (EI) ionization in positive ion mode. The ion source and the interface temperatures were set to 200°C and 250°C, respectively. GC-MS data were acquired and processed using Xcalibur software (v 2.2). FAME derivatives were identified using the National Institute of Standards and Technology library (2014) and quantified in comparison to the amount of the known standard added to each sample.

Protein Quantification: Protein content was quantified using the Bio-Rad DC Protein Assay Kit, employing a modified Lowry assay for colorimetric protein quantification. A standard curve was generated with Bovine Serum Albumin in order to quantify the protein abundance in

the samples. Absorbance readings were generated through use of a UV-spectrophotometer at 750 nm.

III- Results

In order to test if the hydroxyl group is added before or after elongation in the synthesis of lesquerolic acid, fatty acid elongase was inhibited by thiocarbamates EPTC and Diallate. To facilitate the addition of various concentrations of inhibitor to the embryos, the stock of inhibitor was diluted in ethanol as recommended by the manufacturer. Preliminary testing of embryo cultures with and without ethanol (1%) were completed to check the effect of ethanol on embryo growth and biomass composition (data not shown). After ensuring that ethanol had no significant effect on embryo growth, all controls used for the inhibitor tests contained 1% ethanol to provide the most accurate comparison.

For both EPTC and Diallate, the optimal concentration of inhibitor used in the Physaria embryo cultures needed to be determined. The goal was to establish a concentration for each inhibitor at which the percentage of total fatty acid content remained stable and comparable to the control (**Figure 4**), while the percentage of lesquerolic acid was decreased. The culture medium's composition remained the same throughout the different trials, with only the concentration of the inhibitor incrementally increasing each time. Although both inhibitors were thiocarbamates, separate analyses were conducted.

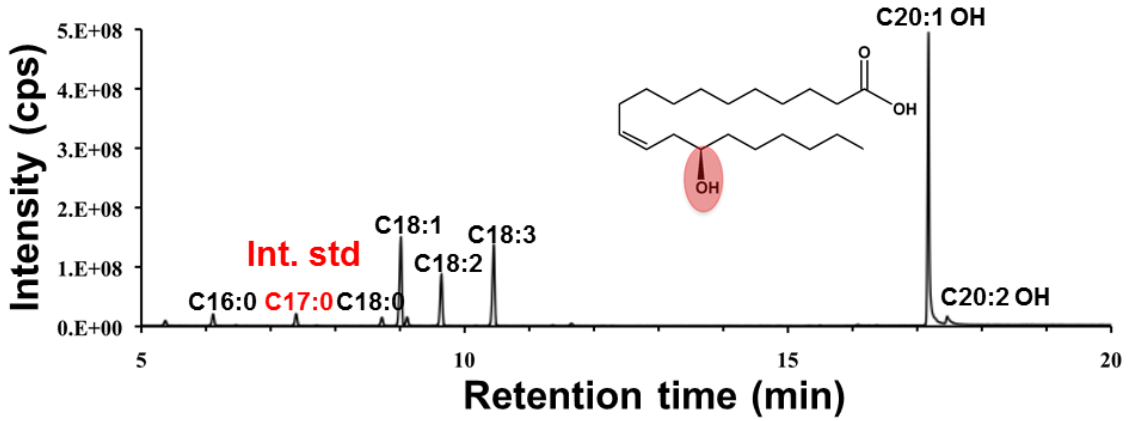


Figure 4. Fatty acid chromatogram from Physaria embryos. Derivatization of fatty acids by methanolic/HCl to be analyzed by gas chromatograph. (Cocuron et al. 2014). C16:0 is Palmitic acid; C18:0, Stearic acid; C18:1, Oleic acid; C18:2, Linoleic acid; C18:3, alpha-Linolenic acid; C20:1 OH, Lesquerolic acid; C20:2 OH, Auricolic acid.

3.1- Effects of EPTC on Biomass and Fatty Acid Composition

Physaria embryos were cultured in presence of various concentrations of EPTC (1, 50, 150, 175, and 200 µM) for 9 days. The dry weight of the harvested embryos was recorded, and then fatty acid and protein content was determined as described in the material and methods. Culturing Physaria embryos in the presence of up to 150 µM EPTC had no visible detrimental impact on embryo growth and development (**Figure 5**).

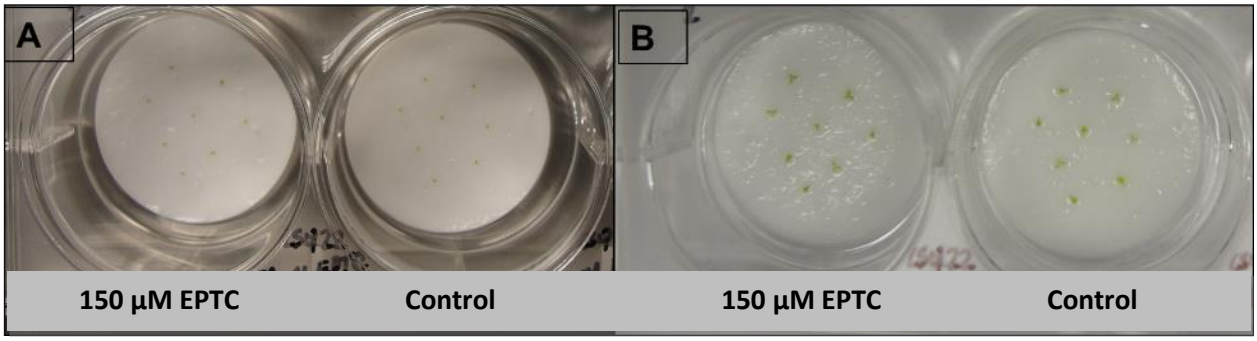


Figure 5. *Physaria* embryo cultures in the presence and absence of EPTC. (A) At the beginning of incubation. 18 DAP (B) After 9 days of incubation. 27 DAP

The dry weight ratios for cultured embryos was close to 1, which was an expected result if there is no difference between weight of embryos grown with inhibitor and that of the control ones (**Figure 6**). **Figure 7** shows that while total fatty acid content remains constant in the presence of EPTC (1 to 200 µM), lesquerolic acid content was observed to be decreasing at the concentration of 150 µM.

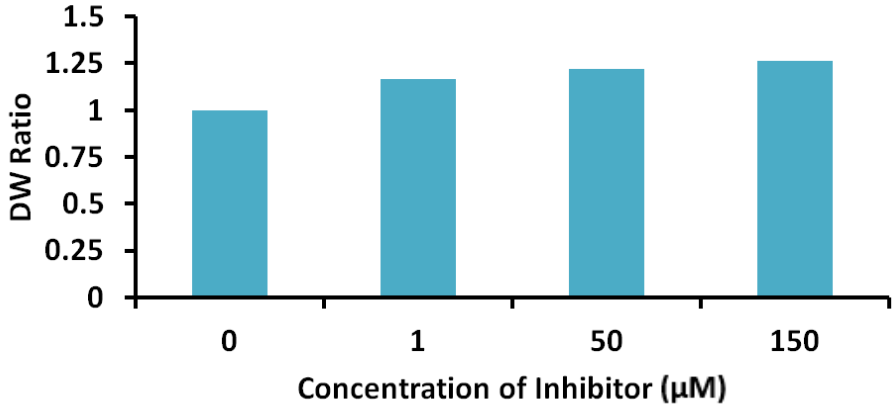


Figure 6. The effect of EPTC on Dry Weight (DW). The dry weight ratios between embryos grown in presence of the inhibitor and the control embryos are presented. Each ratio is an average of two biological replicates.

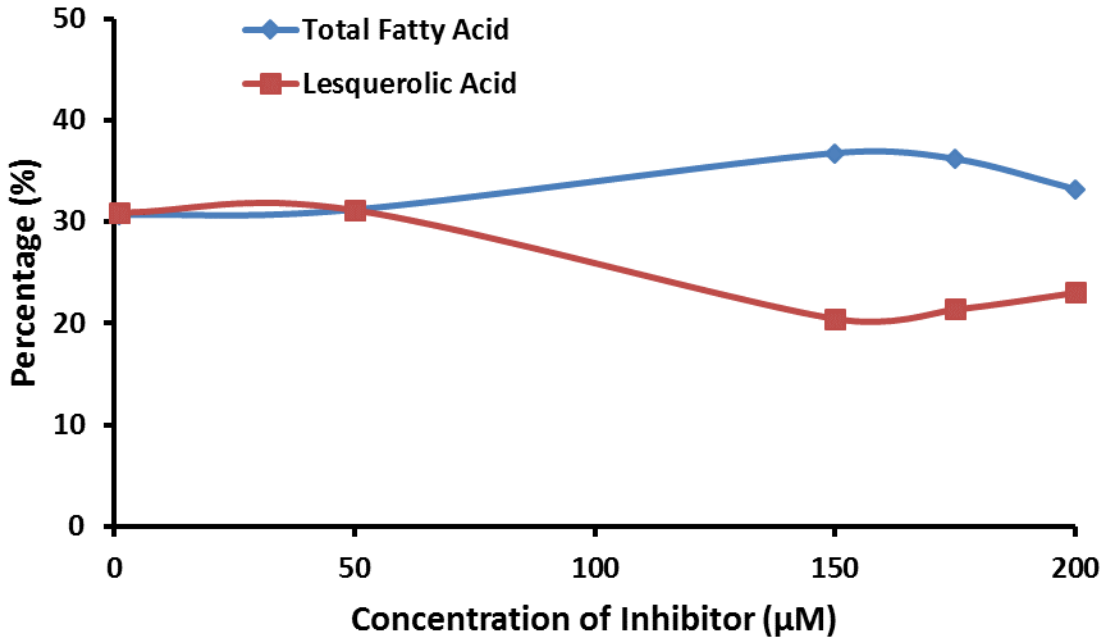
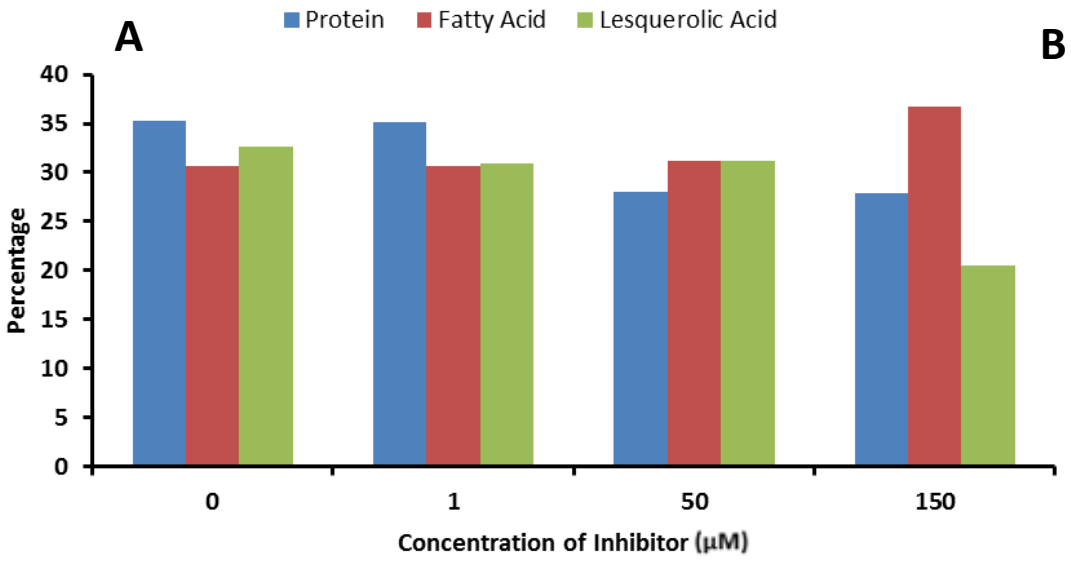


Figure 7. EPTC concentration curve. Embryos were cultured in various doses of EPTC (1, 50, 150, 175, 200 µM). Total fatty acid content is expressed in % w/w. Lesquerolic acid is given as a percent of total fatty acid. Each data point is an average of two biological replicates.

Protein content was found to remain within the same range when embryos were incubated in the presence of 1 to 150 µM of EPTC (**Figure 8A**). Fatty acid composition was determined for the embryos cultured with 150 µM EPTC (**Figure 8B**). There was an increase in the long-chain fatty acids; stearic acid (C18:0) from 2.48% to 5.32% total fatty acid content, and oleic acid (C18:1) from 21.15% to 46.76%, and a decrease in lesquerolic acid (C20:1 OH) from 34.02% to 20.23% (**Figure 8B**). It is important to note that no peak on the chromatogram corresponding to ricinoleic acid (C18:1 OH) or other shorter hydroxylated fatty acids was detected.

150 µM EPTC was found to be a suitable concentration to apply to the cultures without compromising the overall growth and biomass composition of Physaria embryos. As being utilized as a successful inhibitor, EPTC also supported the hypothesis that the hydroxyl group is added after elongation due to the absence of hydroxylated precursors.



B

Fatty Acids	EtOH	EPTC (150 uM)
C16:0	5.21	6.98
C18:0	2.48	5.32
C18:1	21.15	46.76
C18:2	15.69	12.03
C18:3	14.43	8.67
C20:1 OH	34.02	20.23
C20:2 OH	7.02	0.00
total	100.00	100.00

Figure 8. Effect of EPTC on the biomass composition. (A) Comparison of protein, fatty acid, and lesquerolic acid (C20:1 OH) content in embryos grown with different levels of inhibitor. Protein and total fatty acid content are expressed in % w/w. Lesquerolic acid is given as a percent of total fatty acid content. (B) Fatty acid composition of embryos is shown with ethanol (EtOH) compared to 150 µM EPTC. Values given are as a percent of total fatty content. Data shown is an average of two biological replicates.

3.2- Effects of Diallate on Biomass Composition

To further validate the results obtained with EPTC, the thiocarbamate Diallate was supplemented to Physaria embryos grown in culture (**Figure 9**). Various concentrations were tested (1, 50, 100, 120, 135, 150, 175, 200 µM), and embryos were seen with yellow cotyledons

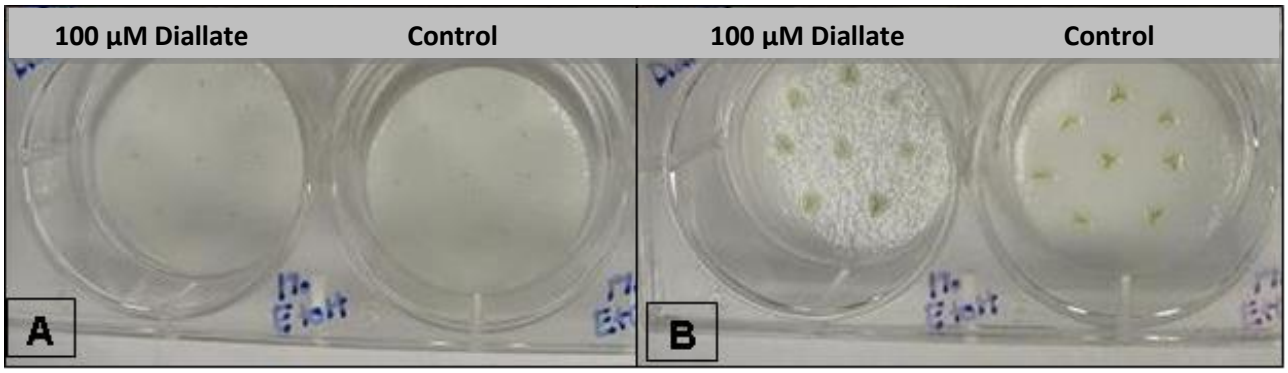


Figure 9. *Physaria* embryo cultures in the presence and absence of Diallate. (A) At the beginning of incubation. 18 DAP (B) After 9 days of incubation. 27 DAP

and translucent hypocotyls when Diallate was introduced at a concentration of 100 µM and higher. This visible effect of Diallate was also reflected in the dry weight of the embryos, which dropped when they were incubated with 100 µM of Diallate (**Figure 10**). Total fatty acid decreased by 20% when embryos were grown with 50 µM of Diallate, and then remained stable for higher concentrations (**Figure 11**). However, lesquerolic acid dropped by 20% and 50% at 50 and 100 µM of Diallate, respectively.

Figure 10. The effect of Diallate on Dry Weight (DW). The dry weight ratios between embryos grown in presence of the inhibitor and the control embryos are presented. Each ratio is an average of two biological replicates.

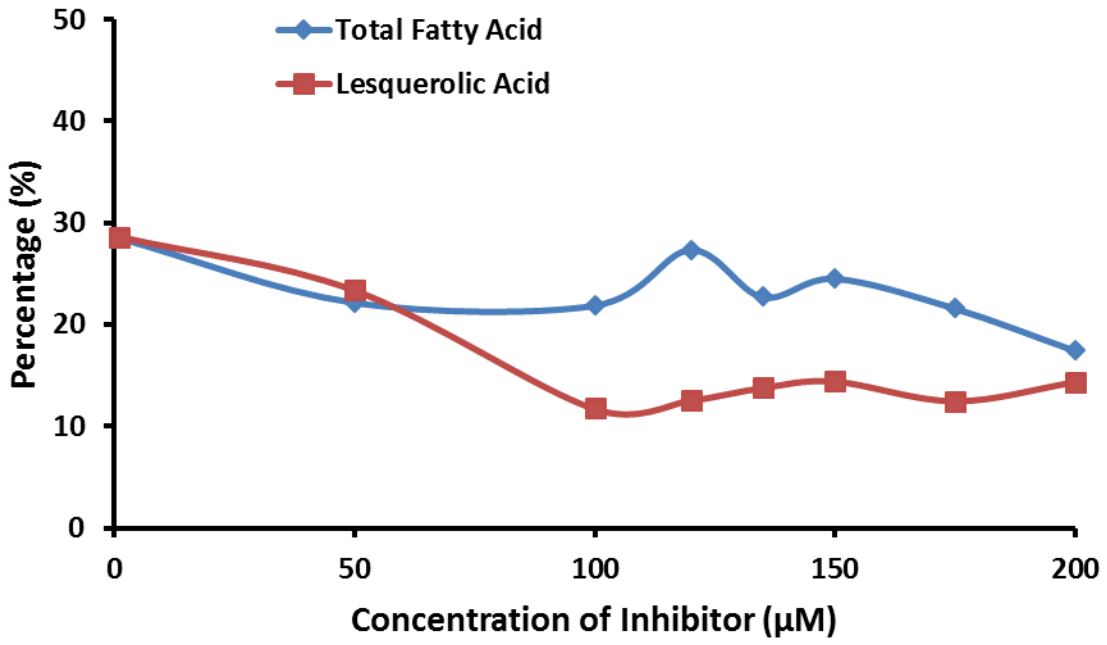
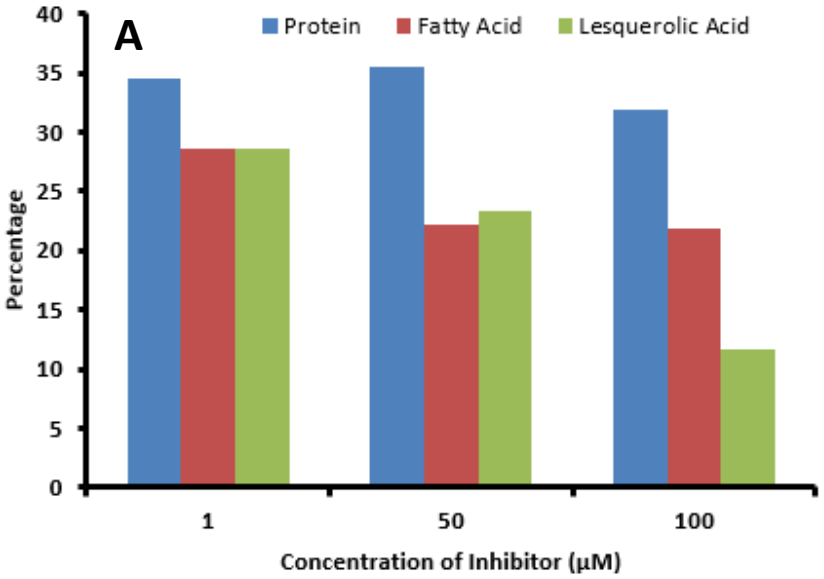


Figure 11. Diallate concentration curve. Embryos were cultured in various doses of Diallate (1, 50, 100, 120, 135, 150, 175, 200 µM). Total fatty acid content is expressed in % w/w. Lesquerolic acid is given as a percent of total fatty acid content. Each data point is an average of two biological replicates.

The protein content was shown to remain stable between 1 and 100 µM Diallate (**Figure 12A**). Fatty acid composition was determined for the embryos grown in the presence of 50 µM Diallate (**Figure 12B**). Lesquerolic acid was shown to decrease from 34.02% to 23.37% of the total fatty acid whereas shorter chain fatty acids increased; oleic acid (C18:1) increased from 21.15% to 25.69% and alpha-linolenic acid (C18:3) increased from 14.43% to 21.68%. As corroborating the previous results, ricinoleic acid and other shorter hydroxylated fatty acids were not present in the extract.

Although not the ideal concentration, 50 µM Diallate was still shown to effectively inhibit the production of lesquerolic acid without compromising the growth of the embryos. With the increased abundance of non-hydroxlyated shorter chain fatty acids, the hypothesis that lesquerolic acid is elongated before hydroxylation was further supported.



B

Fatty Acids	EtOH	Diallate (50 uM)
C16:0	5.21	5.94
C18:0	2.48	3.10
C18:1	21.15	25.69
C18:2	15.69	18.35
C18:3	14.43	21.68
C20:1 OH	34.02	23.37
C20:2 OH	7.02	1.87
total	100.00	100.00

Figure 12. Effect of Diallate on the biomass composition. (A) Comparison of protein, fatty acid, and lesquerolic acid (C20:1 OH) content in embryos grown with different levels of inhibitor. Protein and total fatty acid content are expressed in % (w/w). Lesquerolic acid is given as a percent of total fatty acid content. (B) Fatty acid composition of embryos is shown with ethanol (EtOH) compared to 50 µM Diallate. Values given are as a percent of total fatty content. Data shown is an average of two biological replicates.

IV- Discussion

The aim of this study was to examine the biosynthetic pathway of lesquerolic acid in *Physaria fendleri;* specifically, the order by which elongation of acyl chain and addition of a hydroxyl group takes place. In this study, through use of thiocarbamates that inhibit the elongation of shorter chain fatty acids, it was shown that the hydroxyl group is added after elongation into very long chain fatty acids. Therefore, our findings indicate that the hydroxyl group is added after elongation of the fatty acid chain to lesquerolic acid. Interestingly, these

results are in disagreement with the previously published data that showed ricinoleic acid (C18:1 OH) as the main precursor of lesquerolic acid (Chen et al., 2011, Lu et al., 2006, Lu et al., 2008). A possible explanation for such inconsistency is that previous studies involved inserting a castor fatty acid hydroxylase into different plants such as Camelina and Arabidopsis and not into Physaria. Thus, this may have false implications on the actual pathways that are naturally occurring within Physaria. The data collected from the present study may be more accurate due to the fact that there were no traces of ricinoleic as a precursor for lesquerolic acid in Physaria embryos *in vivo*.

Although this study does not specifically alter the plant in a permanent fashion like gene transformation studies, the use of chemical inhibition could still impact the overall results due to some confounding factors. One possible consequence of utilizing herbicide as in inhibitor is the effect it could have on photosynthesis. With the use of Diallate (100 µM), embryos became yellow and translucent, suggesting that the photosynthetic pathways and chlorophyll content may have been negatively affected. Use of multiple inhibitors of the same family allowed for further analysis of the inhibition of elongases without apparent effect on chlorophyll, considering EPTC was able to reach concentrations above 200 µM without visible yellowing. In order to circumvent this confound entirely, a different type of chemical such as desaturase inhibitors could be utilized to analyze the pathway in the opposite direction. Because desaturase inhibitors only prevent unsaturation of shorter chain fatty acids, it would not inhibit the synthesis of the very long chain fatty acids (Gronwald, 1991). Thus, desaturase inhibitors would potentially increase the proposed lesquerolic acid precursor; oleic acid (C18:1), and provide insight into increasing the flow of oleic acid into lesquerolic acid synthesis.

In addition to increasing the amount of precursors available for lesquerolic acid synthesis, another way to try to produce more HFA would be to introduce all the genes utilized in the lesquerolic acid biosynthetic pathway into a higher-yield crop. One such example employed inserting a fatty acid hydroxylase from the castor plant into camelina (Lu et al., 2008). Although HFAs were produced, they did not accumulate at a high level (~15%). Moreover, the total fatty acid content of the transgenic seeds dropped, preventing the marketing of this plant as an alternative crop. A recent transcriptomic analysis demonstrated that at least 20 genes involved in lipid synthesis appeared to have co-evolved through modulation of both transcriptional abundances and alterations within protein sequences in Physaria embryos (Horn et al., 2016). This co-evolution is thought to channel HFAs into storage lipids (triacylglycerols). This is necessary because HFAs are detrimental to membrane integrity. In order to replicate this pathway into other plant specimens, over 20 genes from Physaria would have to be co-expressed, therefore making this answer an improbable solution. Overall, the simplest resolution would be to improve the output of lesquerolic acid within Physaria itself.

V- Future Work

This research utilizing thiocarbamates as inhibitors of elongases has created preliminary data that advances our knowledge of how lesquerolic acid is synthesized. Now that target concentrations have been obtained, replication of the cultures will need to take place in order to analyze the statistical significance of the different levels of lesquerolic acid, other fatty acids, protein content, as well as starch content. Chlorophyll content will also be analyzed through common extraction practices (Porra et al., 1989, Barnes et al., 1992) to confirm that embryos are still conducting photosynthesis at standard levels in the presence of the inhibitor, since Diallate was causing yellowing at higher concentrations. Desaturase inhibitors such as Chloridazon and

Norflurazon will also be tested to see if they can improve the availability of oleic acid, the suggested precursor of lesquerolic acid (Moon et al., 2001).

Although chemical inhibition work provides insight to the specific formation of lesquerolic acid synthesis, it is also crucial to examine the broader pathway Physaria is utilizing to incorporate HFA into TAG for storage and later use. Work on this subject is being done in the Alonso lab in collaboration with Phil Bates. Briefly, ^{13}C-steady state labeling and ^{14}C-pulse labeling is on-going as a means to quantify the flow of carbon through the central metabolism with the hopes of identifying targets in Physaria that could be utilized for metabolic engineering.

VI- Acknowledgements

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